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In vitro evaluation of *Jatropha curcas* and *Bursera linanoe* resins in the control of phytopathogenic fungi isolated from roselle (*Hibiscus sabdariffa*)

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ABSTRACT

Antecedents/Objectives. Guerrero is an important producer of roselle (*Hibiscus sabdariffa*); therefore, the objective was to evaluate the inhibitory effect of resins *in vitro* using four factors: technique (disc in agar or Kirby Bauer and well in agar), resins (*B. linanoe*, *J. curcas*, mixture of both and Thiabendazole), volume (10, 20 and 30 µL) and phytopathogenic fungi (*C. cassiicola*, *F. oxysporum* and *F. solani*) on the diameter of the inhibition halo.

Materials and Methods. Statistical analysis was performed with a completely randomized factorial design with fixed effects to compare the 72 treatments using the Kruskal-Walis test.

Results. All terms were found to be significant, the main effects of technique, resins, volume and fungi on the diameter of the inhibition halo, but also the double, triple and quadruple interactions.

Conclusion. *B. linanoe* resin showed higher inhibition for *C. cassiicola* and *F. oxysporum*, in the two techniques (agar well and agar disc technique or Kirby Bauer technique), this makes it the resin type with the highest biocontrol potential.

Keywords: inhibition, antifungal, *Corynespora cassiicola, Fusarium oxysporum*, *Fusarium solani.*

Introduction

Roselle (*Hibiscus sabdariffa*) is considered an economically (Chew *et al*., 2024) and medicinally (Takada *et al*., 2024) important species. On an international scale, Mexico ranks seventh as a producer, with 5.14%. The state of Guerrero is the largest producer of roselle in the country. The municipal areas of Ayutla de los Libres and Tecoanapa are the main production areas, followed by Acapulco de Juárez and San Luis Acatlán (SIAP-SADER, 2022). However, the sale price is low, due to the quality and innocuousness since preharvest, due to problems caused mainly by phytopathogenic fungi (Aparicio *et al.,* 2016; Dios-López *et al*., 2011).

Among the most important diseases affecting roselle in Guerrero are the calyx spot induced by *Corynespora cassiicola* (Ortega-Acosta *et al*., 2015a), *Phytophthora parasitica* (Hernández-Morales and Romero-Cova, 1990), *Lasiodiplodia pseudotheobromae, Fusarium incarnatum-equiseti* (Aparicio *et al*., 2016) and other phytopathogenic fungi associated to these symptoms, such as *F. oxysporum*, *F. solani* and *F. incarnatum* (Ortega-Acosta *et al*., 2015b). Recent studies indicate that the roselle spot disease is the main constraint on calyx production, since it displays a 100% incidence in Ayutla and Tecoanapa (Ortega-Acosta *et al.,* 2020).

Studies performed on calyces report that out of 1,470 kg of roselle gathered in Guerrero, 40.8% were of commercial quality, with spots and smut on the tips (equivalent to 588 g of the unit) with calyxes under bad innocuousness conditions. The phytopathogenic fungi related to roselle in storage were *Fusarium* and *Corynespora* (Ruiz-Ramírez *et al*., 2015).

Mexico has a wide variety of temperate climate coniferous and broadleaf tree species (Quiroz y Magaña 2015), with a potential in the use of organic extracts with antifungal effects against phytopathogenic fungi, able to reduce diseases, with favorable effects on the quality of the calyces, including pine nut and linaloe, *Jatropha curcas* and *Bursera linanoe* respectively; they are considered a new organic alternative to reduce the use of chemical fungicides (Del Puerto *et al*., 2014). The aim of this investigation was to evaluate the inhibitory effect of *J. curcas* and *B. linanoe* resins, the mixture of both, and thiabendazole against *C. cassiicola*, *F. oxysporum* and *F. solani* under *in vitro* conditions using the Kirby Bauer and agar well diffusion techniques.

The *J. curcas* resin sample was gathered fresh and the linaloe (*B. linanoe*) resin was taken from the collection of the National Forestry, Agriculture, and Livestock Research Institute (INIFAP), Zacatepec, Morelos experimental field. Both resin samples were taken from the fruit, which were placed in sterile jars with a screw-on lid for their transportation under refrigeration at a 20 °C.

The *C. cassiicola* with Access in GenBank (MF000865) responsible for roselle spot, *F. oxysporum* (KM519188) and *F. solani* (KM519190) associated to the "black

leg" symptom, originate from the roselle cultivation in the state of Guerrero, and were provided by the Laboratory of Plant Health and Safety of the Phytosanitary Institute at the Colegio de Postgraduados-Campus Montecillo.

To determine the *in vitro* effect of the resins on the fungi, the strains were planted in Petri dishes in a Potato-Dextrose-Agar (PDA) medium at room temperature (25 ± 2 °C). After 7 days, it was covered with 10 mL of sterile saline solution at 0.85%, shaken for 3 minutes and rubbed on the surface of the culture, firmly and gently, using a bacteriological loop. The suspension obtained from each one of the strains was poured directly into sterile test tubes and left to rest for 20-30 min. The turbidity of the spore suspension was measured by spectrophotometry, 70 – 75% transmittance at 530 nm, according to the technique by Cermeño and Torres (1998).

For the agar well technique, the technique followed was the one proposed by Moreno-Limón *et al.* (2011). For the Kirby-Bauer technique, the method by Gallardo-Vásquez *et al*. (2019) was followed, using Petri dishes with a PDA culture medium. Separately 10, 20 and 30 µL of the de la spore suspension of each of the fungi was planted, at a concentration of $1x10^1$ with four repetitions.

The Petri dishes were incubated at 25 ± 2 °C for 7 days. Subsequently, the diameter of the radial growth was calculated. The percentage of growth inhibition was calculated considering that the inhibition is the opposite of the growth (Tequida-Meneses *et al*., 2002). The inhibiting effect of the resins was determined to be positive with the appearance of a zone of inhibition around the perforations, which was measured using a 150 x 0.02 mm Fumetax® caliper every 24 h for 7 days.

The minimum inhibitory dose (MID) was determined, which is defined as the lowest concentration of resins that showed no growth in the test tubes. PDA culture medium was used, in which 8 tubes were inoculated with 1 mL of PDA with 10 µL of spore suspension, at a concentration of $1x10^1$, adjusted at 530 nm, 70 – 75% of transmittance (Cermeño and Torres 1998).

Statistical analysis. The sample averages of the studied factors were calculated and added to their standard deviations in parentheses. Box graphs were also created to know their distribution. The experiment followed a completely randomized factorial design with fixed effects. Four factors were studied: technique (T_i) with two levels: agar disk (Kirby Bauer) and agar well diffusion; resins (R_j) with four levels: *B. linanoe*, *J. curcas*, the mixture of both, and thiabendazole, volume (V_k) with three levels: 10, 20 and 30 µL and fungi (H_l) with three species: *C. cassiicola*, *F. oxysporum* and *F. solani,* and the response variable was the diameter of the inhibition halo. The statistical model used was:

$$Y_{ijklt} = \mu + T_i + R_j + V_k + H_l + (TR)_{ij} + (TV)_{ik} + (TH)_{il} + (RV)_{jk} + (RV)_{jl} + (RH)_{kl} + (TRV)_{ijk} + (TRH)_{ijl} + (RVH)_{ikl} + (RVH)_{jkl} + (TRVH)_{ijkl} + \varepsilon_{ijklt}$$

$\varepsilon_{ijklt} \sim IIDN(0, \sigma^2)$; $i = 1, 2$; $j = 1, 2, 3$; $k = 1, 2, 3$; $l = 1,2, 3, 4$; $t = 1, 2, 3$.

The data were analyzed using the R statistical package R (R Core Team, 2020). In the analysis of variance, a significance level of $\alpha = 0.05$ was used. The non-parametric aligned ranks version was used to determine whether the main effects and interactions were significant (Kay *et al*., 2021). To compare the 72 treatments, the non-parametric Kruskal-Wallis test was used.

A modification was performed on the minimum inhibitory concentration (MIC) since, due to the handling of raw and natural samples without lab equipment, their concentrations are unknown; only different doses were handled (Table 1). The *B. linanoe* displayed a better MID in comparison with the rest of the samples (Table 1).

Table 1. Minimum Inhibitory Dose (MID) of *Jatropha curcas*, *Bursera linanoe* resins and Mixture of (*J. curcas* and *B. linanoe*) against the fungi associated with roselle diseases.

Fungi evaluated	Isolation key	Resin of *Jatropha curcas* µL mL^{-1}	Resin of *Bursera linanoe* µL mL^{-1}	Mix of (*J. curcas* and *B. linanoe*) µL mL^{-1}
Fusarium oxysporum	FsrT5	140	4	12
Fusarium solani	Fs34	100	4	12
Corynespora cassiicola	CC33GRO	120	4	8

The 20 treatments show a predominance of the agar well technique, since it appears in 12 treatments; the *B. linanoe* resin treatment is amongst the 20 best treatments, since it appears in nine treatments; the mixture of resins in five treatments, and thiabendazole, in six treatments; the 10 and 20 µL volumes appear in eight and seven treatments respectively, and the fungi *C. cassiicola* and *F. oxysporum* appear in 12 and eight treatments, respectively. The combination of agar well and *B. linanoe* appeared in six of the 20 treatments.

On the opposite end, the group with the lowest means of the inhibition halo diameter contains the five treatments that had a minimum of the inhibition halo diameter of 13 mm (DJcV30Fo, DTV10Fs, DTV30Fs, PTV10Fs and PTV30Fs) and another 14 treatments (DJcV10Cc, DTV20Fo, DJcV30Cc, DTV30Fo, PTV30Fo, DJcV20Cc, DJcV20Fs, PJcV20Fo, DJcV30Fs, PJcV30Fo, DTV20Fs, PTV20Fs, DJcV10Fs, DJcV20Fo), making up a total of 19 treatments. In these treatments, the agar disk technique was predominant, since it appears in 13 treatments; the *J. curcas* resin appears in 10 treatments and thiabendazole, in nine treatments. The 30, 20 and 10 µL volumes appear in eight, seven and four treatments, and the

microorganisms *F. solani, F. oxysporum* and *C. cassiicola* appear in nine, seven and three treatments, respectively (Table 2).

Table 2. Clusters of the 72 treatments after using the Kruskal-Wallis test.

Trto	MDHI	Group*	Trto	MDHI	Group*	Trto	MDHI	Group*
DBlV10Cc	30.0	a	DMV30Cc	11.5	hijklmnop	PJcV20Cc	12.1	efghijklmn
DBlV10Fo	11.3	ijklmnopq	DMV30Fo	21.6	ab	PJcV20Fo	7.4	uvwxy
DBlV10Fs	10.0	klmnopqrs	DMV30Fs	10.6	jklmnopqr	PJcV20Fs	8.3	qrstuvwx
DBlV20Cc	30.0	a	DTV10Cc	12.3	efghijklmn	PJcV30Cc	12.0	efghijklmn
DBlV20Fo	12.7	cdefghijkl	DTV10Fo	9.0	nopqrstuv	PJcV30Fo	7.3	vwxy
DBlV20Fs	9.2	mnopqrstu	DTV10Fs	7.0	y	PJcV30Fs	8.6	opqrstuvw
DBlV30Cc	30.0	a	DTV20Cc	15.0	abcdefgh	PMV10Cc	18.7	abc
DBlV30Fo	9.6	klmnopqrst	DTV20Fo	7.6	stuvwxy	PMV10Fo	12.6	defghijklm
DBlV30Fs	8.7	opqrstuvw	DTV20Fs	7.2	wxy	PMV10Fs	9.4	lmnopqrstu
DJcV10Cc	7.8	rstuvwxy	DTV30Cc	12.0	fghijklmn	PMV20Cc	17.8	abcd
DJcV10Fo	8.1	rstuvwx	DTV30Fo	7.4	tuvwxy	PMV20Fo	15.5	bcdefghi
DJcV10Fs	6.9	wxy	DTV30Fs	7.0	y	PMV20Fs	8.8	opqrstuvw
DJcV20Cc	7.4	tuvwxy	PBlV10Cc	30.0	a	PMV30Cc	13.0	bcdefghijk
DJcV20Fo	7.1	xy	PBlV10Fo	30.0	a	PMV30Fo	12.7	cdefghijkl
DJcV20Fs	7.4	tuvwxy	PBlV10Fs	13.8	bcdefghi	PMV30Fs	13.9	bcdefghij
DJcV30Cc	8.3	stuvwxy	PBlV20Cc	30.0	a	PTV10Cc	30.0	a
DJcV30Fo	7.0	y	PBlV20Fo	30.0	a	PTV10Fo	30.0	a
DJcV30Fs	7.3	uvwxy	PBlV20Fs	13.9	bcdefghi	PTV10Fs	7.0	y
DMV10Cc	16.1	abcdef	PBlV30Cc	30.0	a	PTV20Cc	30.0	a
DMV10Fo	16.2	abcdef	PBlV30Fo	16.7	abcde	PTV20Fo	30.0	a
DMV10Fs	11.4	hijklmnop	PBlV30Fs	13.6	bcdefghij	PTV20Fs	7.2	wxy
DMV20Cc	11.8	ghijklmno	PJcV10Cc	13.5	bcdefghij	PTV30Cc	30.0	a
DMV20Fo	18.3	abcdefg	PJcV10Fo	8.6	pqrstuvw	PTV30Fo	7.4	tuvwxy
DMV20Fs	11.3	ijklmnopq	PJcV10Fs	10.1	klmnopqrs	PTV30Fs	7.0	y

Trto: Treatment; MDHI: means of the inhibition halo diameter, * Means with the same letter are statistically equal. D=Disk in agar or Kirby Bauer, P=agar well, Bl= *B. linanoe*, Jc= *J. curcas*, M= mixture of both resins, T= Thiabendazole, V10=10 µL, V20=20 µL, V30=30 µL, Fo= *F. oxysporum*, Fs= *F. solani,* Cc= *C. cassiicola*.

All factors were found to be significant, including the main effects of the technique, the resins, the volume and the fungi in the diameter of the inhibition halo, as well as the double, triple and quadruple interactions. The *B. linanoe* resin displayed the highest inhibition, and therefore the greatest antifungal effect against *C. cassiicola* and *F. oxysporum*. This antifungal effect can be related to a variety of secondary metabolites found in the plant, such as saponins, phenols, flavonoids and monoterpenic compounds such as the linalyl acetate (Becerra and Noge, 2010; Cruz *et al*., 2016). The antifungal response obtained from *B. linanoe* is consistent with Arellano-Hernández (2018), who in recent studies observed that the resins obtained from different parts of the tree present bacteriostatic and bio-fungicidal properties.

This aligns with Seepe *et al.* (2020), who mentioned that the plant extracts have presented a high antifungal activity against different pathogens of the *Fusarium* genus. The metabolites of the resins, such as linalyl acetate, caryophyllene and undecane, reported in linalool (Cruz e*t al*., 2016).

The case of the *J. curcas* resin did not provide promising results, showing that with the agar well technique, an inhibition halo was obtained in the fungus *C. cassicola*, between 12.0 and 13.5 mm in diameter. Despite Córdova-Albores *et al.* (2016) mentioning that the effect of the *Jatropha curcas* oil has been widely studied, it has been found to have the ability to partially alter the morphology of the mycelium and conidia of *F. oxysporum*, causing morphological and cellular damage, including intense vacuolization. These effects are attributed to the large number of metabolites with antifungal activity found in diverse parts of the plant (Saetae and Suntornsuk, 2010). Rahu *et al.* (2021) also mention that it is crucial that the trials determine the fungicidal effect of the extracts or compounds, since *J. curcas* is a plant with a great potential as an antimicrobial agent.

On the other hand, the mixture of both resins (*B. linanoe* + *J. curcas*) to boost the antifungal effect was lower than expected, presenting inhibition halos of 16.1 to 18.7 mm, greater than those found with the *J. curcas* resin, which presented inhibition halos of 7 to 13.1 mm, though lower than those with *B. linanoe*, with inhibition halos with a diameter of 30 mm. These are marginal means that do not consider the effect of the other three factors studied. Therefore, mixing both resins is not considered a good option. The best minimum inhibitory concentration was for *B. linanoe* (4 µL mL^{-1}), followed by the mixture (12 µL mL^{-1}), and finally, *J. curcas* (140 µL mL^{-1}) (Table 1).

The commercial control thiabendazole displayed control in six of the treatments, presenting an inhibition halo of 30 mm with the agar well technique of the fungi *C. cassiicola* and *F. oxysporum*. This coincides with Medina-Osti *et al*. (2022), who mention that thiabendazole has shown its antifungal action derived from its direct effect on mitosis, which hinders the development of the mycelium, therefore its efficiency in the reduction of the growth of the mycelium in species of *Fusarium*. However, the use of thiabendazole, one of the most efficient fungicides against a wide variety of pathogens, is no longer an effective treatment, although some farmers still use it to control some diseases caused by pathogens (Seepe *et al.,* 2021).

Regarding every fungal species, the largest inhibition halos, with 30 mm, were found in *C. cassiicola*, being one of the most susceptible and with the least resistance, since it displayed greater zones in all the volumes used. *F. oxysporum* also displayed susceptibility with a 30 mm inhibition halo, presenting larger zones in the fungal volumes of 10 and 20 µL. This suggests that the use of these resins may be a viable alternative against the roselle leaf and calyx spots. These findings

coincide with those by Moreno-Limón *et al*. (2011), who evaluated different *Aspergillus* and *Penicillium* genera spore suspensions, observing a high variability in their analysis. However, the reports on *C. cassiicola* in roselle are scarce in the state of Guerrero (Ortega-Acosta *et al*., 2015a). It is therefore important to continue researching to understand the specific action mechanisms of the *Bursera linanoe* resin.

No significant differences were found in the evaluations of the volumes of fungi used, since the largest inhibition halo diameters were consistently observed in the volumes of 10, 20 and 30 µL. The *B. linanoe* resin, obtained from the fruit of the tree, is considered a promising and efficient alternative as a biofungicide in the control of *C. cassiicola*, *F. oxysporum* and *F. solani*, according to the *in vitro* results. Therefore, investing in the development of medicinal plant-based products for the control of crop diseases caused by pathogens is a growing sector that should be considered and developed (Seepe *et al*., 2021).

Reducing the use of conventional synthetic fungicides by incorporating natural effective products is a crucial step towards a sustainable agricultural production. Nevertheless, it is crucial to understand the action mechanism of these resins and evaluate their effectiveness under field conditions.

It was found that all terms were significant, including the main effects of technique, resins, fungal volume and fungal species on the diameter of the inhibition halo, as well as the double, triple and quadruple interactions.

The results of this *in vitro* investigation show that the *B. linanoe* resin exhibited greater inhibition for *C. cassiicola* and *F. oxysporum* in both techniques (the agar well technique and agar disk or the Kirby Bauer technique), making it the type of resin with the greatest biocontrol potential.

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